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Disruption of dopamine circuitry following exposure to the organochlorine insecticide endosulfan: Implications for neurological disease

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Abstract

Disruption of dopamine circuitry following exposure to the organochlorine insecticide endosulfan: Implications for neurological disease
By Lauren Shapiro

Background: Endosulfan is an organochlorine insecticide and acaricide used to protect a wide variety of foods, plants, trees and shrubs. Although endosulfan was banned in 2011, its ability to persist in the environment and human body poses a threat to public health. Epidemiological studies have suggested that endosulfan exposure contributes to neurological disease, however there is minimal research that addresses the cellular mechanism or target of endosulfan in the nervous system.

Objective: This current study is designed to determine whether endosulfan exposure disturbs the dopaminergic system and contributes to the onset of neurological disorders.

Methods: Endosulfan toxicity was first assessed in the SH-SY5Y dopaminergic cell line using a cytotoxicity assay, and then in primary culture neurons taken from the ventral mesencephalon. Finally, the impact of endosulfan was evaluated using mice treated with 1 mg/kg of endosulfan for 30 days, mimicking potential human exposure. Animals were challenged with MPTP to evaluate endosulfan's impact on the dopaminergic system in the striatum. Immunohistochemistry and immunoblotting were performed to determine the effect of endosulfan on various neuronal proteins.

Results: Endosulfan caused toxicity in SH-SY5Y cells in a dose dependent fashion (LD_{50}=400 uM). Primary culture neurons were drastically more sensitive to endosulfan as the LD_{50} in this model was 20 uM. The animal exposure study confirmed previous findings that MPTP decreases striatal dopamine transporter (DAT) and tyrosine hydroxylase (TH) levels by 66% and 31% respectively. Assessment of cortical dopaminergic markers revealed novel results that indicate endosulfan elicits a 35% decrease in DAT and a 50% decrease in TH. Finally, endosulfan exposure caused a 40% increase in cortical synapsin expression.

Discussion: Endosulfan's impact on DAT and TH indicates that exposure disrupts the dopaminergic system in the cortex. Synapsin is a protein essential for transmission in allneural systems, suggesting that an increase in cortical synapsin implicates changes to normal neural transmission in the cortex. Future studies must determine behavioral and neurochemical changes induced by endosulfan to fully elucidate its effect on the brain, and its association to neurological disease such as autism, ADHD and schizophrenia.

Disruption of dopamine circuitry following exposure to the organochlorine insecticide endosulfan: Implications for neurological disease

By

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B.S. Bates College, 2010

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INTRODUCTION

Parkinson's Disease

Parkinson's disease is the second most common neurodegenerative disorder associated with age. The progressive disease impacts approximately 2% of the population over 65 and about 4% of the population over 80 (Corti et al 2005, Mutch et al 1986). Clinical manifestations of Parkinson's disease include slowed movements, tremors and muscular rigidity. Neuronal loss occurs in the nigrostriatal pathway, where the cell bodies in the substantia nigra pars compacta project to the caudate and putamen. Furthermore, patients tend to exhibit a loss of smell, blurred vision, orthostatic hypertension, abnormal sleep patterns, nausea, constipation, bladder disturbances, fatigue, and approximately 40% shown signs of depression (Chaudhuri et al 2006). Such symptoms result from the loss of dopamine and dopamine producing neurons in the nigrostriatal pathway of the basal ganglia (Corti et al 2005). Clinical diagnosis is usually made based upon clinical symptoms as brain imaging techniques do not always provide conclusive evidence for the condition (Davie 2008). When parkinsonian symptoms are first detected, it is believed that most patients have an 80% decrease in dopamine in the putamen and a 60% loss of neurons in the substantia nigra pars compacta (Dauer & Przedborski 2003). The dopamine degeneration is specific to the nigrostriatal pathway, as the neurons in the ventral tegmental area of the midbrain that comprise the mesolimbic pathway are relatively spared (Uhl et al 1985). It is important to note that although neuronal degeneration occurs naturally as a part of aging, the degeneration that occurs during Parkinson's is distinct and selective to the nigrostriatal pathway. In addition to the fact that Parkinson's degeneration is specific to the motor pathway, there is also more

terminal loss than cell body loss, which is not seen in the normal aging process. Such observation also indicates that striatal terminals are more involved and perhaps more vulnerable than the cell bodies in the neural mechanism of the disease (Bernheimer et al 1973).

Anatomy

Parkinson's disease is a movement disorder caused by dysfunction of the basal ganglia. The basal ganglia are located bilaterally in the midbrain and are comprised of a number of subcortical nuclei such as the striatum (caudate and putamen) and the globus pallidus. The basal ganglia is involved in complex motor movements, associative learning, planning, and emotion which are all mediated by the dopaminergic system. Complex connections with numerous other parts of the brain allow for the basal ganglia to be involved in such varied tasks (Obeso et al 2008). For example, one of the most important inputs to the basal ganglia comes from the substantia nigra pars compacta. The substantia nigra pars compacta sends both excitatory and inhibitory messages mediated by dopamine to the caudate nucleus and putamen which are responsible for regulating movement (Carlson 2010).

Pathways. Dopamine is involved in mediating a variety of neurological functions including movement, attention, learning, and reward. Furthermore, there are three major dopaminergic systems in the brain that are responsible for facilitating these behaviors. The nigrostriatal system involves neurons in the substantia nigra and project to the caudate nucleus, putamen of the basal ganglia. It is this pathway that is responsible for movement and is dysfunctional in Parkinson's disease. The mesolimbic system is comprised of neurons from the ventral tegmental area and project to the nucleus

accumbens, the amygdala and the hippocampus. This pathway is specifically involved in reward and reinforcing behaviors that are a part of the learning process. Finally, the mesocortical system also starts in the ventral tegmental area but sends signals to the prefrontal cortex. The mesocortical system facilitates short-term memory, planning, and problem solving as well as other executive functions (Carlson 2010, Fuxe 1985).

Treatment

The most widely used treatment is Levodopa, which is the precursor to dopamine. Levodopa is highly effective in improving motor symptoms in the early phase of treatment, however long term therapy results in severe side effects, specifically unwanted movements known as dyskinesias. Dyskinesias caused by levodopa increase at an average rate of 10% per year after treatment begins. It is believed that spontaneous stimulation of dopamine receptors is the cause of such motor movements but researchers have had difficulties mitigating the side effects (Davie 2008). Other treatments include dopamine agonists, which, also work to increase the available dopamine in the brain, and MAO-B inhibitors that slow the breakdown of the dopamine and allow for extended transmission.

Dopamine

Synthesis. Dopamine, norepinephrine, epinephrine and serotonin are called monoamines because they all have a single amine group. More specifically, dopamine, norepinephrine and epinephrine are catacholamines as they are similarly structured and their synthesis involves many of the same enzymes. The biosynthesis of dopamine begins with the amino acid tyrosine, which is a staple in many proteins consumed and thus ubiquitous in the body. Tyrosine hydoxylase converts tyrosine into Levo-DOPA (L-

DOPA) by adding a hydroxyl group. A carboxyl group is then cleaved from L-DOPA by DOPA decarboxylase to make dopamine (Figure 1). In dopaminergic neurons, the biosynthesis pathway stops at the production of dopamine; however, norepinephrine-secreting neurons have an additional enzyme, dopamine β-hydroxylase, which converts dopamine into norepinephrine through the addition of a hydroxyl group (Carlson 2010).

Synpatic transmission. Following synthesis, dopamine is then transported via the vesicular monoamine transporter (VMAT2) into vesicles where it is stored until secreted. Once the action potential propagates the length of the axon and reaches the terminal, the vesicles fuse with the terminal membrane and dump the dopamine into the space between neurons known as the synapse. VMAT2 serves a crucial role in synaptic transmission, as free-floating dopamine that is not packaged in synaptic vesicles is susceptible to breakdown and inactivation by enzymes in the axon terminal. Furthermore, only packaged dopamine is released into the synapse to propagate the signal (Figure 1).

Once dopamine is in the synapse, it is eligible to bind to post-synaptic receptors on the surrounding neurons. Activation of receptors has a variety of effects including the initiation of a complex intracellular protein cascades as mentioned previously. The signal is terminated in two common ways. Dopamine can be broken down by enzymes in the synapse or it can be brought back up into the pre-synaptic neuron through the process of reuptake. In reuptake, dopamine is transported into the terminal by the dopamine transporter (DAT) where it is recycled and reused (Figure 1) (Carlson 2010).

Receptors. The two most common dopaminergic receptor families are D_1 and D_2 receptors. D_1 receptors are primarily found post-synaptically and stimulation of these receptors elicits a complex intracellular protein cascade that involves increased

production of cyclic AMP, an important secondary messenger. Unlike D_1 receptors, D_2 receptors have the exact opposite effect on cyclic AMP, as D_2 stimulation results in a decrease of cyclic AMP. Furthermore, D_2 receptors are found both pre-synaptically as well as post-synaptically. D_3, D_4, and D_5 receptors are scattered throughout the brain and their activation also initiates intracellular protein cascades involving cyclic AMP (Carlson 2010).

Neural Mechanism

Although the exact mechanism of neurodegeneration in Parkinson's disease is not fully understood, it is known that proteasomal dysfunction, mitochondrial dysfunction and oxidative stress contribute to neuronal loss.

Proteasomal dysfunction. It is possible that misfolded proteins interfere with normal dopaminergic function, providing one possible mechanism for neurochemical symptoms of PD. In Parkinson's disease, the protein aggregates, known as Lewy bodies, are comprised of various misfolded proteins. It is unclear whether the protein aggregates themselves are toxic or if they sequester proteins involved in cell survival, which in turn reduces the neurons' defense (Dauer & Przedborski 2003). Although less likely, it is also possible that aggregates themselves are not the toxic component, but rather protein aggregates are a neuron's way of trapping toxic chemicals that have entered the cell (Cummings et al., 1999). Researchers have also noticed that α-synuclein, the primary component of Lewy bodies, aggregates more easily when it is oxidized, suggesting that oxidation is the precursor for protein aggregation (Giasson et al 2002).

Further evidence to support the notion that protesomal dysfunction contributes to the neural mechanism of Parkinson's comes from patients with a hereditary form of the

disease. These individuals have genetic mutations for proteins that are found in the Lewy body inclusions. Potentially the mutation causes the proteins to misfold in a way that initiates aggregates with other surrounding proteins (Bussell & Eliezer 2001).

Mitochondrial dysfunction. It is well documented that the mitochondrial complex I is dysfunctional in Parkinson's patients (Greenamyre et al 2001). Researchers first became interested in mitochondrial function in PD patients following the discovery that MPTP, a synthetic drug that mimics the Parkinsonian pathology, is a complex I inhibitor (Nicklas et al 1987). Interfering with complex I releases harmful oxidative species into the cytosol and prevents the cell from sufficiently producing energy. Complex I activity is abnormal in the brain and platelets of PD patients, suggesting that mitochondrial dysfunction is not confined solely to the central nervous system (Schapira et al 1990).

Oxidative stress. Under normal circumstances oxidants are released as a byproduct of respiration, and during dopamine synthesis. Complex I dysfunction increases the amount of reactive oxygen species (ROS) that are released, resulting in severe oxidative stress. Such ROS cause cellular damage through oxidative stress because they are highly reactive and interfere with lipid, protein and nucleic acid functioning and also increase the number of misfolded proteins (Dauer & Przedborski 2003). Although the entire mechanism for neuronal death is not fully understood, it is evident that oxidative stress, caused by complex I dysfunction can cause an unhealthy cellular state the eventually results is neuronal death.

Neural degeneration and Lewy body inclusions are also found in other neural circuits besides the dopaminergic nigrostriatal pathway, such as the locus coeruleus, the dorsal raphe and the nucleus basalis of Meynert. Incorporating these regions into the

neuropathology of Parkinson's indicates that the serotonergic, cholinergic and noradrenergic systems. These regions play smaller roles in the pathology of the disease and their involvement is not fully understood (Hornykiewicz & Kish 1987).

Gene-environment interaction

Genetic components. Although only about 5% of PD cases appear to have a genetic linkage, several genes responsible for some of PD's neural manifestations have been identified. Of particular relevance are SNCA, PARK2, PTEN-induced putative kinase 1, DJ-1 and Leucine-rich repeat kinase 2 (LRRK2). Mutations in these genes and their variants account for the majority of identified mutations in sporadic Parkinson's Disease. The SNCA gene codes for α-synuclein and is believed to play a role in neurotransmitter release. LRRK2 codes for the protein LRRK2 and although its function is unknown, it is the most mutated PD gene identified thus far. PARK2 codes for parkin, a protein found in lewy bodies, that is believed to target proteins for degradation and regulate mitochondrial function. The PINK1 protein is involved in oxidative stress response and mitochondrial functioning. Finally, DJ-1 is a redox sensor that is involved in the oxidative stress response as well (Nuytemans et al 2010).

Although it appears that the identified genes account for a number of the neurological manifestations of the disease, Parkinson's is not entirely genetic. A study of Parkinson's disease in twins conducted in 1999 provided invaluable insight to the cause of the disease. In the case where one monozygotic twin was diagnosed with Parkinson's disease prior to the age of 51, the other twin was six times more likely to also develop Parkinson's compared to dizygotic twins. Furthermore, when a monozygotic twin was diagnosed with Parkinson's disease after the age of 51, the other twin was equally as

likely as developing Parkinson's as the dizygotic twins. Such evidence suggests there are etiological differences between early onset and late onset Parkinson's. Additionally, the results clearly indicate that the disease is not entirely genetic (Tanner & Ben-Shlomo 1999). Following this study, researchers began investigating possible environmental risk factors for Parkinson's disease that could account for the non-genetic component of the disease.

Environmental components. The first major breakthrough in understanding the neural mechanisms behind Parkinson's disease occurred in 1983 when drug users mistakenly injected themselves with MPTP instead of the synthetic opiate analog MPPP. The drug users developed a motor condition that very similarly resembled Parkinson's disease. MPP+ is the active ingredient of MPTP, and is taken into the neurons via dopamine transporters. Once inside the neurons MPP+ inhibits complex I of the mitochondria causing damage through oxidative stress. Furthermore, MPTP also induced Lewy-body like structures with α-synuclein inclusions similar to those observed in the brains of Parkinson's patients (Langston et al 1983).

Additional research has shown that dopaminergic neurons are most severely damaged from MPP+ suggesting that they are more susceptible to the adverse effects of complex I inhibition than other systems (Dauer & Przedborski 2003). Besides MPP+, rotenone and paraquat, both pesticides, have similar patterns of causing selective degeneration to dopamine neurons in the nigrostriatal pathway (Betarbet et al 2000, McCormack et al 2002). Such evidence supports the notion that nongenetic factors may also contribute to the onset of Parkinson's disease.

Identified risk factors

Metals. Chronic exposure to various metals such as copper, iron, lead, manganese and mercury can potentially contribute to development of neurological disease, as metals tend to target the central nervous system. Individuals are typically exposed to such metals through either inhalation or ingestion. Manganese, iron, mercury and occasionally copper, persist in fumes emitted in various occupational settings. Lead, copper and mercury can contaminate food and water sources (Caudle et al 2012).

Chronic exposure to metals may contribute to the development of Parkinson's disease, as iron, manganese, copper, and mercury have all been reported to cause oxidative stress, which is believed to be a major component of the neural mechanism that causes dopaminergic depletion in the basal ganglia (Caudle et al 2012). Furthermore, iron and copper accumulate in the substantia nigra and interact with α-synuclein in-vitro in addition to causing oxidative stress through various enzymatic reactions (Dexter et al 1989, Earle 1968, Uversky et al 2001). α-Synuclein aggregates in dopaminergic neurons contributing to their dysfunction. Finally, lead and mercury have been shown to disrupt dopaminergic transmission. Although the mechanism in which lead and mercury impede dopamine function in unknown, epidemiological studies provide further evidence to support the hypothesis that exposure contributes to abnormal dopamine transmission (Caudle et al 2012, Gorell et al 1999, Weisskopf et al 2010).

Pesticides. Epidemiological studies have found increased rates of Parkinson's disease in individuals exposed to pesticides through occupational or residential settings (Roberts et al 2007).

Fungicides. Fungicides are used to prevent mold and fungus from growing on crops. They typically have a metal or sulfur base and although they are not known to produce severe toxic effects independently, there is evidence that some fungicides, such as Maneb can exacerbate the neurotoxic effects of other more potent pesticides like paraquat (Hatcher et al 2008, Thiruchelvam et al 2000)

Herbicides. Bipyridyl paraquat is used globally, and has been deemed a risk factor for Parkinson's disease because it has a structure similar to that of MPTP, a synthetic drug that elicits Parkinsonian-like symptoms (Snyder & D'Amato 1985). Both epidemiological and animal studies have been unable to consistently demonstrate the direct link between paraquat exposure and the development of Parkinson's disease (Hatcher et al 2008).

Insecticides. Research on neurotoxicity and pesticides is primarily on rotenone. Rotenone is a naturally occurring chemical that is most frequently used in organic agriculture. It acts to inhibit complex I, increasing oxidative stress. Further studies indicate that exposure to rotenone selectively damages dopaminergic neurons and causes the protein inclusions that resemble lewy bodies (Betarbet et al 2000, Hatcher et al 2008).

Organochlorines. Organochlorines are a family of highly chlorinated insecticides that were used in large quantities in the 1940s. A combination of widespread usage and severe bioaccumulation in soil, water and human tissue resulted in high levels of exposure and deposition. In the 1970s, there was a large-scale ban that sought to reduce mass treatment of agricultural products with organochlorines. For the past several decades, global organizations have continued to ban usage of specific organochlorine products, however traces of the chemicals remain in the environment (Sharma et al

2010). In some parts of the world, acute exposures to organochlorines still occur in occupational settings, while more commonly, chronic exposures result from food consumption (Jones & Miller 2008). Numerous cohort and case control studies have indicated that elevated levels of organochlorines in the body are associated with higher incidence of Parkinson's disease (Sharma et al 2010).

Dieldrin is a cyclodiene organochlorine that is of particular interest in the study of pesticides' role in Parkinson's disease as despite its discontinued use, researchers found dieldrin residues in post-mortem brain samples of Parkinson's patients (Corrigan et al 2000). In animal models, dieldrin has been found to decrease striatal dopamine and increase dopamine transporter (DAT) levels as well as the vesicular monoamine transporter (VMAT2) levels which is responsible for packing dopamine prior to release (Hatcher et al 2008). Furthermore, developmental exposure to dieldrin increased the neurotoxicity of MPTP when administered later in life. Dieldrin inhibits normal mitochondrial function, increases ROS and quinone levels and initiates apoptosis of dopamine neurons (Kitazawa et al 2003). Finally, effects elicited by dieldrin appear to only impact the dopaminergic system (Richardson et al 2006). Such results provide evidence to suggest that dieldrin alters the dopaminergic system in such a way that it is more susceptible to developing Parkinson's disease.

Heptachlor, another cyclodiene pesticide designed for both household and agricultural use, has also been shown to target the dopaminergic system. Just like dieldrin, heptachlor has been banned for a number of decades, however it bioaccumulates in both the environment so individuals continue to be exposed through the consumption of agricultural goods. Animal studies have shown that heptachlor administered during

development increases VMAT2, DAT and TH levels (Caudle et al 2005). Although VMAT2 levels are elevated following exposure, heptachlor actually hindered VMAT2 transport (Miller et al 1999).

These studies show that organochlorine pesticides cause toxicity to the dopaminergic system by increasing dopaminergic markers, such as TH, DAT and VMAT (Caudle et al 2005, Richardson et al 2006). Endosulfan is another organochlorine insecticide, however, unlike heptachlor and dieldrin, it is still used worldwide. There is little known about endosulfan's impact on the dopaminergic system but further research on its mechanism could clarify the role that organochlorines play in the development of Parkinson's disease.

Current study

Endosulfan is a synthetic organochlorine insecticide and acaricide used to protect a wide variety of foods, plants, trees and shrubs. Despite it's known ability to act as an endocrine disruptor and to cause neurotoxic effects; endosulfan is used ubiquitously world-wide (Silva & Gammon 2009). Endosulfan is fat soluble and accumulates in the body of humans and animals that are exposed to contaminated air, water or food products (Agency for Toxic Substances & Disease Registry 2001). Similarly, endosulfan accumulates in the soil, as it takes years for it to break down. The most common route of exposure is through consumption of contaminated oils, fats and vegetables. People are also exposed by smoking cigarettes with tobacco grown in endosulfan-containing soil. Furthermore, individuals involved in manufacturing endosulfan can become exposed to high concentrations of the chemical, as can those applying endosulfan in an agricultural setting. People living near or working in hazard waste sites may also become exposed.

Individuals can limit their exposure to endosulfan by washing agricultural products prior to consumption, limiting tobacco intake and avoiding manufacturing and hazardous waste sites that may contain endosulfan (Agency for Toxic Substances & Disease Registry 2001).

Endosulfan, when consumed in high quantities, causes neurological symptoms and occasionally death. Little is known of the physiological symptoms of endosulfan exposure in humans; however, short-term exposure in animals appears to damage the stomach, liver and kidneys. Long-term exposure severely damages the kidneys and the reproductive system and may affect the immune system (Agency for Toxic Substances & Disease Registry 2001).

Other organochlorines have been long removed from the global market due to their ability to bioaccumulate in both terrestrial and aquatic ecosystems and their potential link to neurological disorders such as Parkinson's disease (Caudle et al 2005, Centers for Disease Control and Prevention 2010). In May 2011, the Stockholm Convention on Persistant Organic Pollutants finally agreed to add endosulfan to the list of banned chemicals (Environmental News Service 2011) The Environmental Protection Agency has initiated a slow phase-out of the endosulfan that restricts its use, but does not call for full elimination until 2016 (U.S. Environmental Protection Agency 2010).

Minimal research has been done to uncover the mechanisms by which endosulfan exerts its neurotoxic effects. Like heptachlor, endosulfan is a GABA(a) receptor antagonist. Electrophysiological studies determined that GABA(a) receptor antagonists excite dopaminergic neurons in the substantia nigra pars compacta (Paladini & Tepper 1999). Given that the substantia nigra pars compacta is also the region involved in

degeneration of Parkinson's disease, it is plausible that endosulfan may disturb the dopaminergic system, like heptachlor and other organochlorines that are associated with the onset of Parkinson's disease. Furthermore, endosulfan may also disrupt dopaminergic function in other regions, including the cortex, where it serves as an important modulator of neurotransmission. Indeed, a previous study demonstrated that endosulfan inhibits both acquisition and retention of memory, both of which are mediated by dopamine in the frontal cortex (Lakshmana & Raju 1994). An epidemiological study investigating maternal residence and autism spectrum disorders concluded that mothers living near field sites sprayed with either endosulfan, dicofol or a combination of the two were 6.1 times more likely to have a child with autism than women who were not highly exposed (Roberts et al 2007). Therefore, this current study is designed to determine whether endosulfan, like other organochlorines, disrupts the dopaminergic system and whether exposure may contribute to the onset of neurological deficits or disorders.

Public health relevance

As modern medicine allows individuals to live longer lives, it is inevitable that the prevalence of age-related diseases will increase worldwide. Unfortunately, Parkinson's disease and other neurological diseases are often debilitating conditions that severely reduce the quality of life of those individuals suffering from them. In order for researchers and doctors to develop an effective treatment with limited side effects, the mechanisms for the disease must be better understood. Furthermore, it is important to identify risk factors for neurological disease so that the global burden does not continue to increase.

METHOD

Cytotoxicity assay

Human derived dopaminergic neuroblastoma SH-SY5Y cells were cultured with DMEM/F12 media (1:1 mixture) with 10% FBS and 1% Penicillin Streptomycin on Poly-D-lysine coated plates. Cells were incubated at 37°C with 95% oxygen and 5% carbon dioxide. Media was changed every 2-3 days and cells were split when confluence exceeded 90%. Cells were seeded at a density of 30,000 cells per well in a 96 well plate and exposed to DMSO, 50 uM, 100 uM, 200 uM, 300 uM, 400 uM and 500 uM of endosulfan I in .02% DMSO (AccuStandard, New Haven CT) for 24 hours. Cells were incubated in WST-1 (Roche Applied Science, Indianapolis, IN) for 45 minutes and absorbences were determined by the Biotek Epoch plate reader (Winooski, VT).

Primary culture

Culture preparation. The primary culture procedure was taken with modifications from (Brewer & Torricelli 2007) and personal communication with Tansey Lab, Emory. Ventral mesencephalon cells from mouse pups (postnatal day 1-3) were extracted and placed in a petri dish with 50 ml Hibernate A (Brainbits, Springfield, IL) + 1 ml B27 (Invitrogen, Carlsbad, CA) on ice. Tissue of interest was first digested in papain (Sigma, St. Louis, MO) and Dispase II (Roche Applied Science, Indianapolis, IN) and then triturated to mechanically dissociate. Trituration was repeated to obtain a supernatant of 6 mls and was then centrifuged at 1100 rpm for 2 minutes. Supernatant was removed and pellet was resuspended in 2.5ml of plating media comprised of Neurobasal-A (Invitrogen, Carlsbad, CA), FBS and Penn/Strep 100x. Cells were counted and diluted to a concentration of about 1 x 10^6 cells/ml. Cultures grew for 7 days and were then treated

with DMSO, 5 uM, 10 uM, 15 uM 20 uM and 30 uM endosulfan I for 24 hours.

Stereology. To prepare cultures for staining, tissue was fixed first in 2% paraformaldehyde solution and then in 4% paraformaldehyde. Tissue was blocked in a 10% NGS, .1% Triton X-100, 1% BSA, 1X TBS solution, rinsed with TTBS (1X TBS + 0.1% Triton) and then incubated with both rabbit anti-TH and mouse anti- MAP2 primary antibodies at 4°C overnight. The second day, tissue was again rinsed in TTBS and then incubated in fluorescent secondary antibodies in a 1% NGS, 1% BSA, .1% Triton X-100 TBS solution at a dilution of 1:400. Cultures were mounted on slides with fluorescent mounting media and TH+ and MAP2+ neurons were quantified using unbiased stereologic software (Stereo Investigator, MBF, Williston,VT).

Animal study

Dosing. Procedure was performed as previously described by (Caudle et al 2006). Twenty, eight-week-old male C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed in a 12:12 light/dark colony with food and water available *ad libitum.* All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health) and approved by the Institutional Animal Care and Use Committee at Emory University. Mice were orally gavaged with either 0 or 1 mg/kg of endosulfan I dissolved in corn oil vehicle daily for 30 days. This concentration is well below the LD_{50} and demonstrated to not elicit any overt health effects including weight loss, death or convulsions. Oral exposure was chosen because humans are commonly exposed to endosulfan through the consumption of contaminated water and agricultural products.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP injections were administered based upon (Richardson et al 2006)**.** Immediately following the 30-day dosing regimen, mice were given two subcutaneous injections of saline or 10 mg/kg of MPTP (Sigma, St.Louis, MO) 12 hours apart. Animals were sacrificed one week following second MPTP injection and tissue from the striatum and cortex was collected for analysis.

Western Blots. Procedure was performed as previously described by (Caudle et al 2006). Tissue from striatum and cortex was briefly homogenized to prepare for Western immunoblotting. Samples were run on a polyacrylamide gel electrophoresis on a NuPAGE 10% Bis-Tris gel (Invitrogen, Carlsbad, CA). Samples were electrophoretically transferred to a polyvinylidene difluoride membrane. Non-specific sites were blocked with 7.5% nonfat dry milk in a Tris-buffered saline solution (135mM NaCL, 2.5mM KCL, 50mM Tris, and 0.1% Tween 20, pH 7.4). Antibodies for DAT (rat), TH (mouse), VMAT2 (rabbit), dopamine D2 receptor (rabbit), vAChT (rabbit), vGLUT (rabbit), GAD67 (mouse), GAT1 (rabbit), vGAT (rabbit), GABAa receptor (rabbit), synaptophysin (rabbit) and synapsin (mouse) to determine the expression of each protein in both striatal and cortical tissue. Actin levels were determined to confirm equal loading. Membranes were incubated overnight in monoclonal primaries for each of the proteins of interest. Primaries were detected using anti-rat, mouse and rabbit horseradish peroxidase secondary antibodies and enhanced chemiluminescence. Luminescence signal was captured on a Biorad Chemidoc. Blots were stripped using Pierce Stripping Buffer (Rockforld IL) and reprobed for additional protein expression.

Immunohistochemistry. Procedure was performed as previously described in Caudle et al., 2006. Brains were serially sliced at 40um on a freezing microtome. Tissue was first rinsed in TBS and then with 3% hydrogen peroxide to prepare for blocking. Tissue was blocked for 1 hour in a 10% normal goal serum and 0.15% Triton TBS solution. Sections were incubated overnight in a rabbit anti-TH or rat anti-DAT primary antibody**.** Primary antibody was detected with a biotinylated goat-anti rabbit or goat anti-rat secondary antibody. Equal amounts of avidin and biotin were used to amplify immunohistological staining (VectaStain Elite Avitin Biotin Complex kit, Vector Laboratories, Burlingame, CA). Finally, tissue was stained with 3'3'Diaminobenzidine (DAB) solution comprised of urea and DAB for 1-3 minutes (SigmaFast DAB, Sigma, St.Louis MO).

Statistical analysis: Image software was used to quantify western blots. Statistical analyses were performed using Graphpad Prism software. Differences between groups were determined using a one-way analysis of variance with Newman-Keuls post hoc test.

RESULTS

Endosulfan toxicity was tested in the SH-SY5Y neuroblastoma cell line and in mesencephalic primary culture. Twenty, eight-week-old male C57BL/6J mice were given 1 mg/kg endosulfan or vehicle via oral gavage for 30 days followed by an MPTP challenge. The three levels of the experiment were designed to examine the effects of endosulfan on the dopaminergic system. Administration of endosulfan daily for 30 days resulted in no overt signs of toxicity, including no significant changes in body weight and no change in appearance or gross behavior (data not shown).

Analysis of endosulfan toxicity in SH-SY5Y cell line

Endosulfan cytotoxicity in the SH-SY5Y dopaminergic neuroblastoma cell line was assessed in a WST-1 assay. Cells treated with 50 uM, 100 uM, 200 uM, 300 uM, 400 uM, and 500 uM of endosulfan showed a dose dependent significant increase in toxicity compared to DMSO. All p values <.001 as shown, demonstrating endosulfan as a neurotoxicant (Figure 2).

Analysis of endosulfan toxicity in primary culture

In order to determine if endosulfan is selectively toxic to the dopaminergic system, endosulfan toxicity was assessed in primary cultures isolated from the dopamine-rich ventral mesencephalon. Stereological software was used to count TH stained neurons and MAP2 stained neurons. A significant decrease in vitality in cells treated with 20 uM of endosulfan compared to cells treated with DMSO, 5 uM, 10 uM or 15 uM of endosulfan; all p values <.05 (Figure 3a,b). Additionally, images of TH+ and MAP2+ neurons treated with 20 uM endosulfan show increased toxicity (decreased neurite quantity and length) compared to TH+ and MAP2+ neurons treated with DMSO (Figure 3c). These results suggest that 20 uM of endosulfan is toxic to dopaminergic as well as to other neurotransmitter systems. At 30 uM there were no viable MAP2+ or TH+ neurons.

Analysis of endosulfan toxicity in adult mice

Although the main intention of this study was to determine the impact of endosulfan on the dopaminergic system, it is possible that endosulfan exerts its effects on various transmitter systems. Western blot analysis of tissue from animals treated with endosulfan determined the impact of endosulfan on the dopaminergic, GABAergic, cholinergic and glutamatergic systems using various antibodies. The effects of

endosulfan on the dopaminergic system were obtained using DAT (70 kDA), TH (60 kDA), VMAT2 (75 kDA) and the dopamine D2 receptor (50 kDA); the GABAergic system using GAT1 (72 kDA), GAD67 (67kDA), vGAT (57 kDA) and GABA(a) receptor (51 kDA); synaptic integrity using synaptophysin (40 kDA) and synapsin (80 kDA) and other transport systems using vAChT (cholinergic) (70 kDA) and vGLUT (glutamatergic) (60 kDA). Actin (42 kDa) was used to ensure even loading and to normalize during quantification.

Striatal expression. In the striatum, there is a significant decrease in DAT expression in CTL/MPTP ($p<.001$) and endosulfan/MPTP ($p <.001$), indicating an effect of MPTP on striatal DAT. A similar result was observed in striatal TH expression in CTL/MPTP ($p<.01$) and endosulfan/MPTP ($p<.01$) (Figure 3). There was no effect of endosulfan on either DAT or TH. Neither MPTP nor endosulfan impacted VMAT2 expression in the striatum (Figure 4). Furthermore, there were no significant effects of MPTP or endosulfan in any of the GABAergic markers (Figure 6), vAChT, VGLUT or synaptophysin and synapsin (Figure 7).

Immunohistochemical analysis. To further verify the results from the western blots, immunohistochemistry was performed on striatal brain slices to determine the impact of endosulfan exposure on the dopaminergic system. Slices were stained for TH and DAT (Figure 5). Although no quantitative analysis was performed, visual assessment clearly confirms that endosulfan does not impact TH or DAT expression in the striatum, however MPTP decreases the expression of both proteins.

Cortical expression. In contrast to the striatum, endosulfan had a much more robust effect on the dopaminergic system in the cortex. There is a significant decrease in

DAT expression between controls and endosulfan, CTL/MPTP and endosulfan/MPTP (all p-values <.01) indicating an effect of endosulfan as well as an effect of MPTP on cortical DAT expression. Similarly, there is a significant decrease in TH expression between control and endosulfan, CTL/MPTP and endosulfan/MPTP (all p-values <.01) indicating an effect of endosulfan and MPTP on cortical TH expression (Figure 8). Additionally, there was an increase in cortical synapsin expression in endosulfan, CTL/MPTP and endosulfan/MPTP treated mice compared to controls (all p-values<.05) indicating an effect of both endosulfan and MPTP on cortical synapsin expression (Figure 10). Neither MPTP nor endosulfan impacted cortical VMAT2, synaptophysin, vAChT, vGLUT, or any GABAergic marker expression (Figures 8-10).

DISCUSSION

This study provides strong evidence to support that endosulfan is neurotoxic and has a detrimental impact on expression of important components involved in cortical neurotransmission. The cytotoxicity experiment demonstrated that endosulfan is toxic to a dopaminergic, neuroblastoma-derived cell line (SH-SY5Y) in a dose dependent fashion. This finding validates previous studies that have also investigated the effects of endosulfan in SH-SY5Y cells (Jia & Misra 2007a, Jia & Misra 2007b).

Assessing endosulfan toxicity in primary culture cells from the ventral mesencephalon demonstrated that primary culture cells are drastically more sensitive to endosulfan than the SH-SY5Y cells. In the cytotoxicity experiment, 400 uM of endosulfan was the LD_{50}, the concentration in which 50% of the cells died, whereas in primary culture, 20 uM of endsoulfan was the LD_{50}. Primary culture neurons are likely more sensitive to endosulfan because they are fully functioning neurons with a complex

protein composition, so there are more mechanisms for endosulfan to target and subsequently disrupt. SH-SY5Y cells on the other hand are much simpler so they are not as susceptible to endosulfan's toxicity.

Stereological analysis of the primary culture experiments, indicated endosulfan was equally toxic to dopaminergic neurons and neurons of other transmitter systems suggesting no selective toxicity. However, analysis consisted of counting neural cell bodies without taking into account dendritic branching or dendrite length. Dystrophic neurites and neurite loss was evident in a visual assessment of primary cultures treated with lower concentrations of endosulfan, but neurite toxicity was not quantified in these experiments. In vitro research has shown that the blockade of the GABA(a) receptor by GABA(a) antagonists like endosulfan, actually initiates firing of dopaminergic neurons in the substantia nigra pars compacta (Paladini & Tepper 1999). Future studies should seek to determine the impact of endosulfan exposure on both GABAergic neurons as well as on dendritic branching, as these results might provide further insight to endosulfan's mechanism of toxicity.

The animal study, which involved exposing animals to 1 mg/kg of endosulfan for 30 days, was designed to mimic exposure humans might experience. MPTP was administered as a challenge following the dosing paradigm, as MPTP is known to cause Parkinsonian like symptoms. It was believed that exposure to MPTP would serve to exacerbate subtle dopaminergic dysfunction that may not be detected through our assessment. MPTP exposure alone caused a decrease of both tyrosine hydroxylase (TH) and the dopamine transporter (DAT) by 31% and 66% respectively. These results determined from western blot analysis were further supported by immunohistochemistry

findings. MPTP's ability to decrease both DAT and TH in the striatum is no surprise as a similar finding has been previously reported (Jakowec et al 2004). The fact that endosulfan, either alone or when given with MPTP did not effect the striatum suggests that the striatum may not be the major target for endosulfan's effects. Furthermore, these results indicate that endosulfan exposure does not severely contribute to the development of Parkinson's as previously hypothesized.

In contrast to the striatum, endosulfan had a dramatic effect on the dopamine system in the cortex. Exposure to endosulfan alone resulted in significant reductions of cortical TH and DAT expression by 35% and 50% respectively. These changes in dopamine markers are interesting given the fact that GABA(a) antagonists like endosulfan stimulate dopamine release and may contribute to alterations in dopamine transmission in the cortex. When endosulfan was given to pregnant mice, offspring had reduced GABA concentrations in the prefrontal cortex, while dopamine concentrations were not altered. Future studies should seek to quantify levels of dopamine in the cortex of adult mice.

Dopamine mediates a wide range of cortical functions including, but not limited to, attention, learning, working memory, object recognition and planned movements (Carlson 2010). Although it is unclear which of these executive functions is affected by endosulfan's disruption of cortical dopamine transmission, these results provide a foundation for the future investigation of behavioral changes induced by endosulfan exposure. More specifically, TH and DAT are down-regulated in autism (Ernst et al 1997) and contribute to abnormal cortical transmission in other dopamine-mediate diseases such as autism, ADHD and schizophrenia (Meisenzahl et al 2007, Volkow et al

2007). Indeed, a recent epidemiological study demonstrated a 6-fold increase in autism spectrum disorder diagnosis in children of mothers highly exposed to endosulfan (Roberts et al 2007).

Endosulfan exposure also induced a 40% increase in synapsin in the cortex. Synapsin plays a critical role in neurotransmission by regulating the vesicle pool in the nerve terminal. Synapsin is able to reversibly tether the vesicles containing neurotransmitter to the cytoskeleton of the axon terminal. Phosphorylation of synapsin results in the release of the vesicles from the cytoskeleton, allowing vesicles to fuse to the synaptic membrane and pour the neurotransmitter into the synapse (Greengard et al 1993). Studies using synapsin knock-out mice have provided further insight to the role that synapsin plays in neurotransmission. In the neurons of mice without synapsin there were fewer vesicles in the recycling pool, and fewer vesicles fusing to the synaptic membrane to release neurotransmitter (Ryan et al 1996). There is little research on the effect that up-regulation of synapsin has on neurotransmission, however, it is likely that the increase due to endosulfan exposure is a compensatory response to abnormalities in the dopaminergic system (Hilfiker et al 1999). It is currently unclear which types of neurons have an increase of synapsin in their terminals, so future studies should determine where exactly the up-regulation of synapsin is occurring.

This study yields invaluable findings that provide novel insight into the toxicity caused by endosulfan. Results implicate that the cortical dopaminergic system is the most vulnerable to endosulfan toxicity, and more specifically indicate that endosulfan disrupts key proteins involved in neurotransmission. Although future studies must further

elucidate the mechanism by which endosulfan disturbs neurotransmission, these results identify cortical dopaminergic system of primary concern.

These results are environmentally relevant, as the in vitro and animal studies were designed to assess the toxicity of endosulfan in a continual exposure paradigm similar to one humans might experience. Results from the current study support the notion that endosulfan toxicity could contribute to alterations in neurological function, however additional research must be done to determine behavioral and neurochemical changes induced by endosulfan to fully elucidate the underlying mechanisms of action and subsequent neurobehavioral disorders. Finally, there have been no previous studies investigating the effects of environmental toxicants on the cortex of the brain. Future neurotoxicology studies should seek to assess the impact of exposure on the cortex to generate a better understanding of how environmental contaminants, besides endosulfan, could contribute to neurological diseases that involve cortical dysfunction.

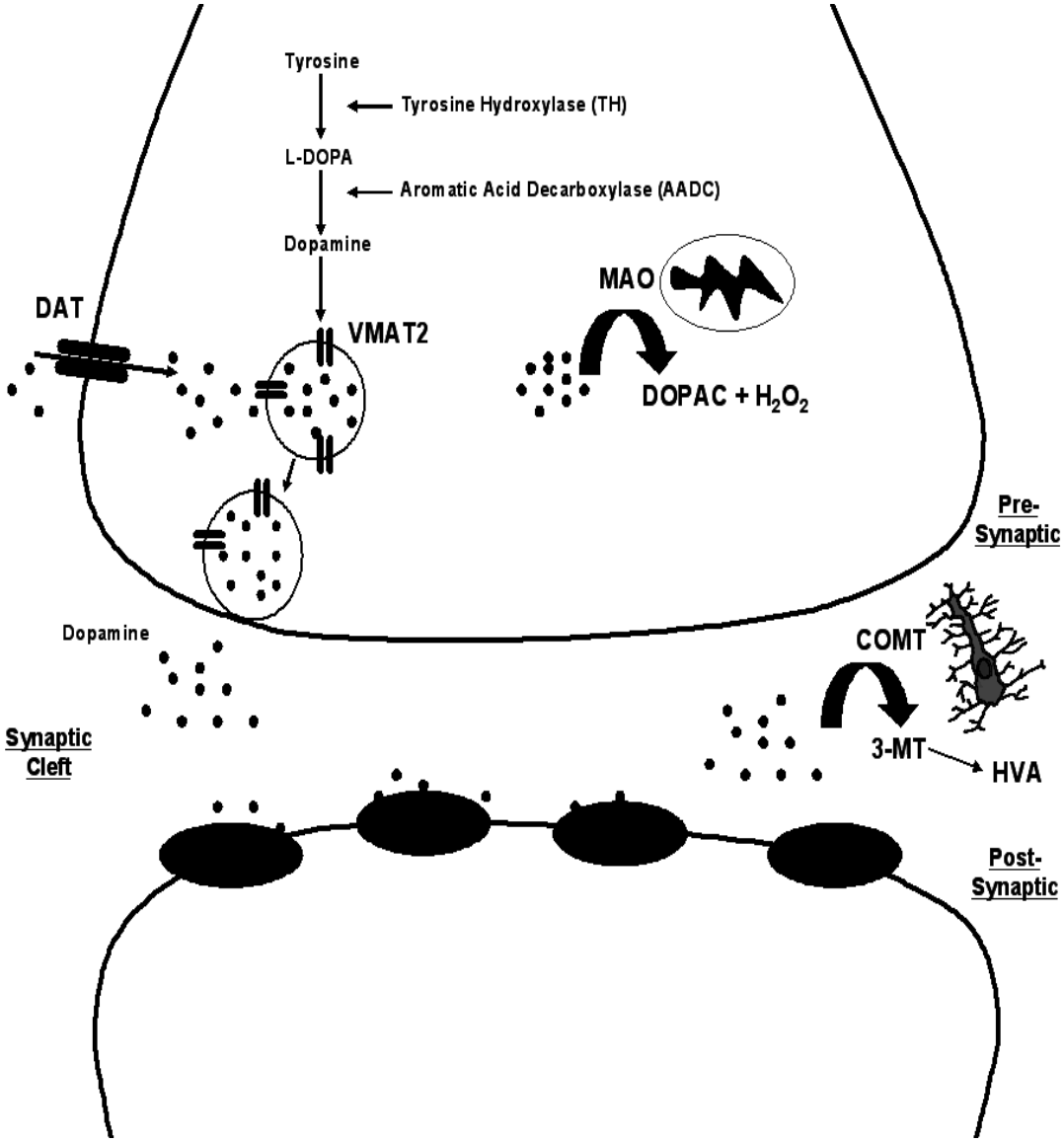

Figure 1. Synthesis, Packaging and Secretion.

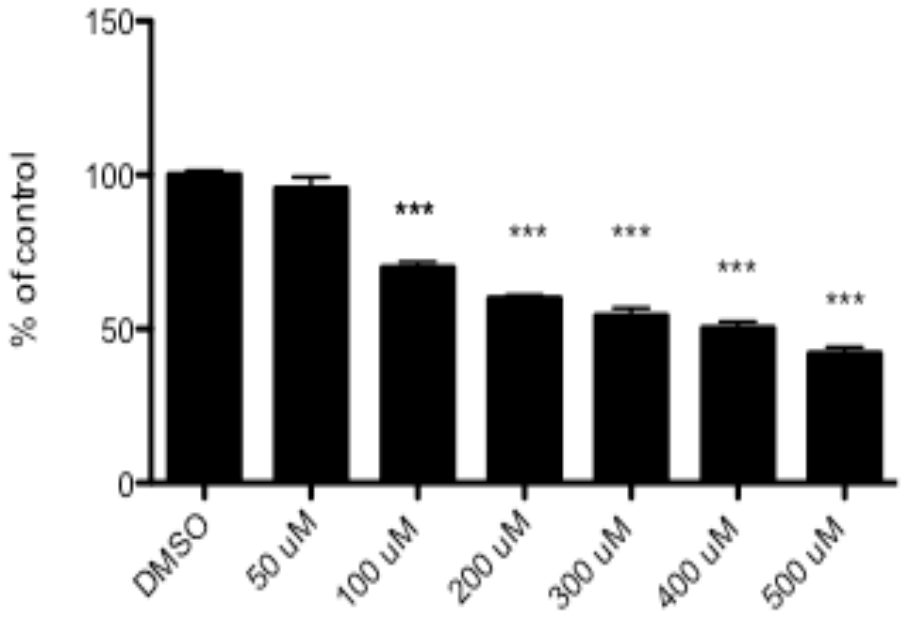

Figure 2. Endosulfan toxicity in SH-SY5Y cells. Cells treated with DMSO, 50 uM, 100 uM, 200 uM 300 uM, 400 uM, 500 uM endosulfan show a dose related response in WST-1 assay (*** indicate p< .001). Asterisks indicate significance compared to DMSO, however each concentration above 100 is also significant compared to all others. One-way ANOVA and Neuman-Keuls multiple comparison post-test was used for statistical analyses; n=27-30 for DMSO, 100 uM, 200 uM, 300 uM, 400 uM and n=10-13 for 50 uM and 500 uM. Data represent % control (DMSO) $\pm$ SEM.

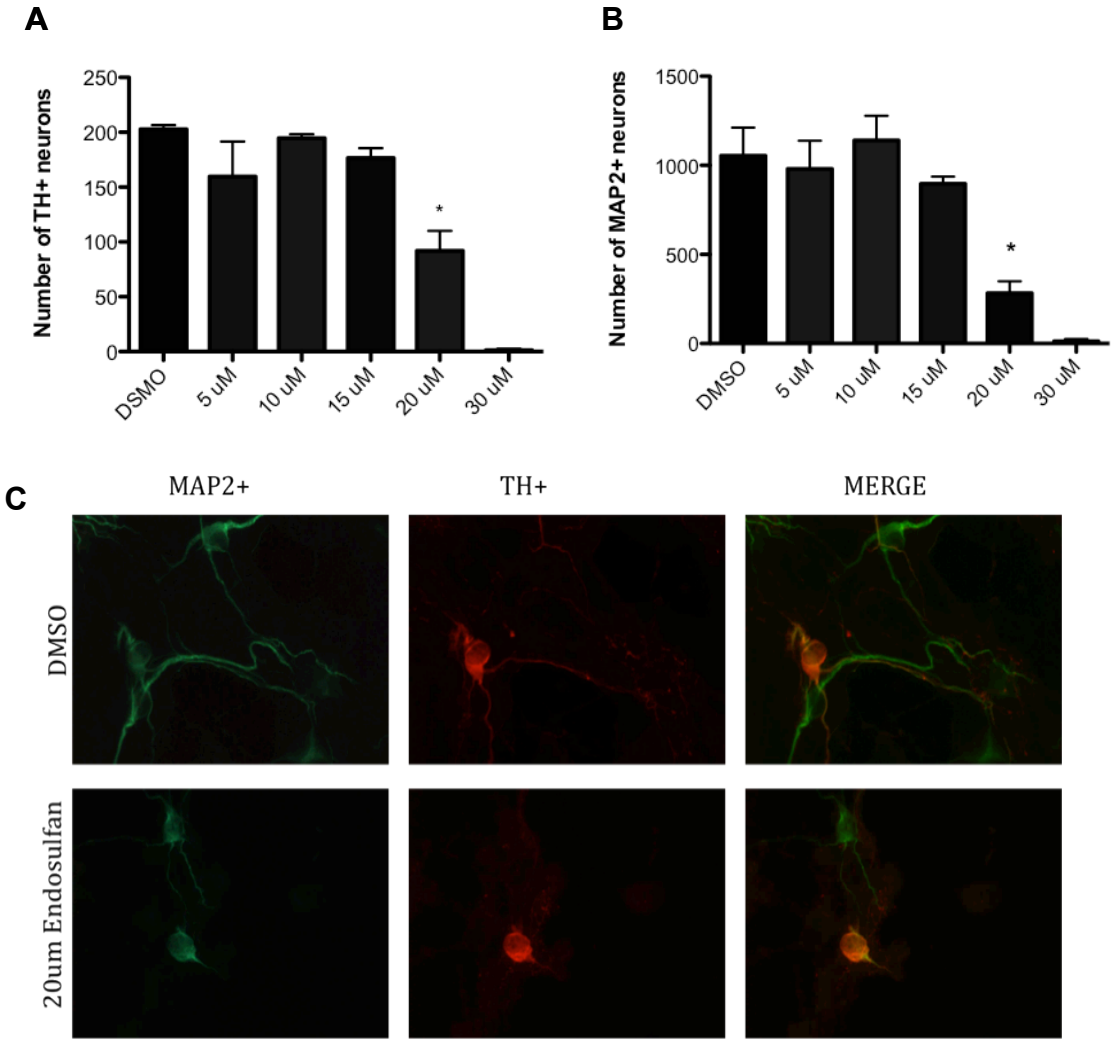

Figure 3. Endosulfan toxicity in ventral mesencephalic neurons. Mesencephalic primary culture cells treated for 24 hours with DMSO, 5 uM, 10 uM, 15 uM, 20 uM, or 30 uM Endosulfan and stained with antibody for TH and MAP2 indicate 20 uM of endosulfan is toxic. TH staining highlights dopaminergic neurons only and MAP2 stains all neurons. A. Stereological counting shows a loss of dopaminergeric neurons at 20 uM of endosulfan (* indicates p< .05). One-way ANOVA and Newman-Keuls multiple comparison post-test was used for statistical analysis; n=3-4 per each treatment group. Data represent average $\pm$ SEM. B. Stereological counting shows a loss of MAP2 stained neurons at 20 uM of endosulfan (* indicates p< .05). One-way ANOVA and Newman-Keuls multiple comparison post-test was used for statistical analysis; n=3-4 per each treatment group. Data represent average $\pm$ SEM. C. Fluorescent TH and MAP2 antibodies on DMSO and 20uM endosulfan primary culture neurons.

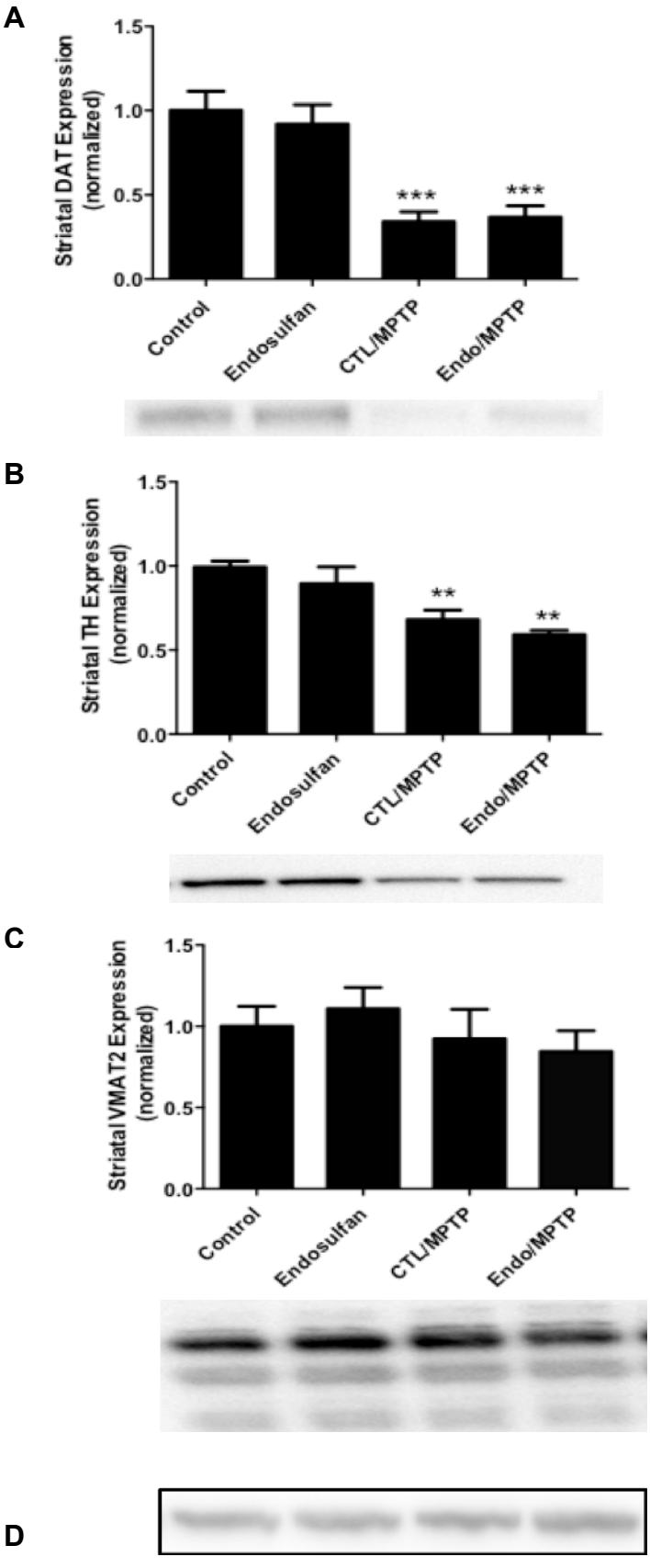
A
Striatal DAT Expression (normalized)
1.5
1.0
0.5
0.0
***
***
Control
Endosulfan
CTL/MPTP
Endo/MPTP
B
Striatal TH Expression (normalized)
1.5
1.0
0.5
0.0
**
**
Control
Endosulfan
CTL/MPTP
Endo/MPTP
C
Striatal VMAT2 Expression (normalized)
1.5
1.0
0.5
0.0
Control
Endosulfan
CTL/MPTP
Endo/MPTP
D

Figure 4. Western blot analysis of striatal dopaminergic markers. Mice exposed to endosulfan I at concentrations of 0 or 1 mg/kg for 30 days and challenged with 2 doses of either saline or 10 mg/kg of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) show a reduction in some striatal dopaminergic protein levels. One-way ANOVA and Newman-Keuls multiple comparison post-test was used for statistical analyses; n=5 per each treatment group. Data represent average $\pm$ SEM normalized to actin. A. Western blot analysis shows a significant reduction in striatal DAT levels in animals exposed to MPTP (*** indicates $p < .001$). B. Western blot analysis shows a significant reduction in striatal TH levels in animals exposed to MPTP (** indicates $p < 01$). Representative Western blots of TH and actin to ensure equal protein loading. C. Western blot analysis shows a no change in striatal VMAT2 levels. D. Representative Western blot of actin to ensure equal loading.

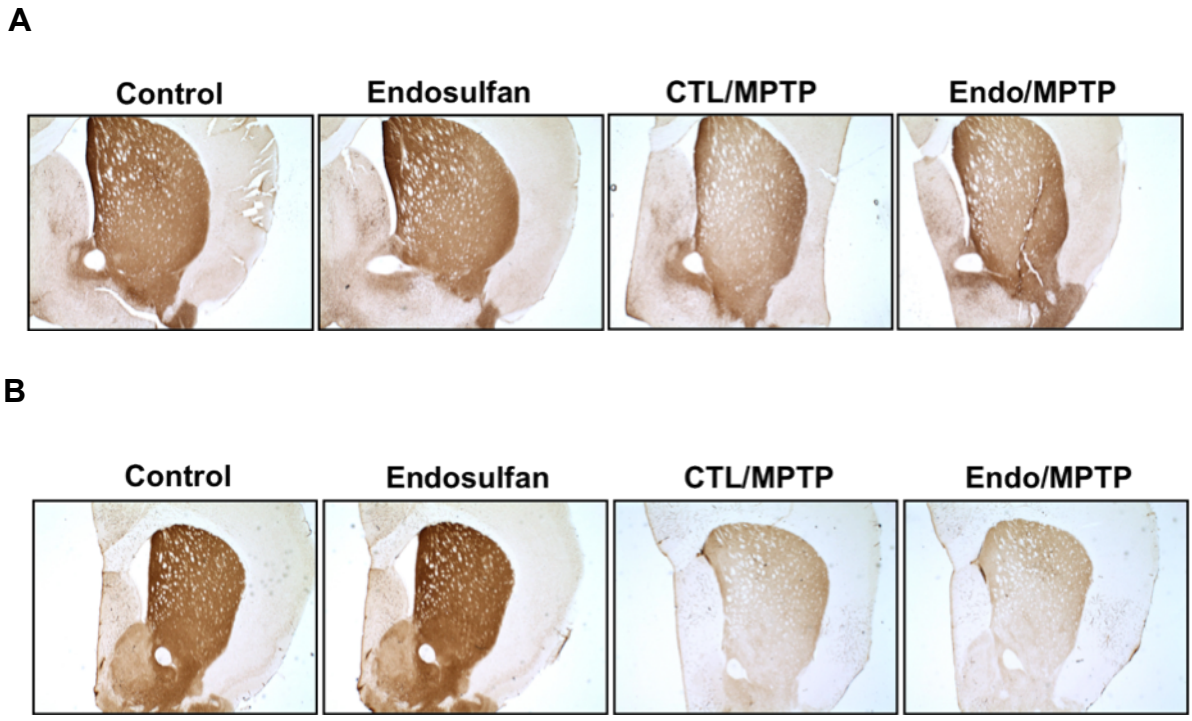

Figure 5. Immunohistochemistry of striatal dopaminergic markers Visual represation of immunohistochemistry of striatal brain slices. Mice were exposed to endosulfan I at concentrations of 0 or 1 mg/kg for 30 days and challenged with 2 doses of either saline or 10 mg/kg of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). A. DAB staining for TH is darker in slices of control and endosulfan treated mice than MPTP treated mice, indicating MPTP decreases TH levels in the striatum. B. DAB staining for DAT is darker in slices of control and endosulfan treated mice than MPTP treated mice, indicating MPTP decreases DAT levels in the striatum.

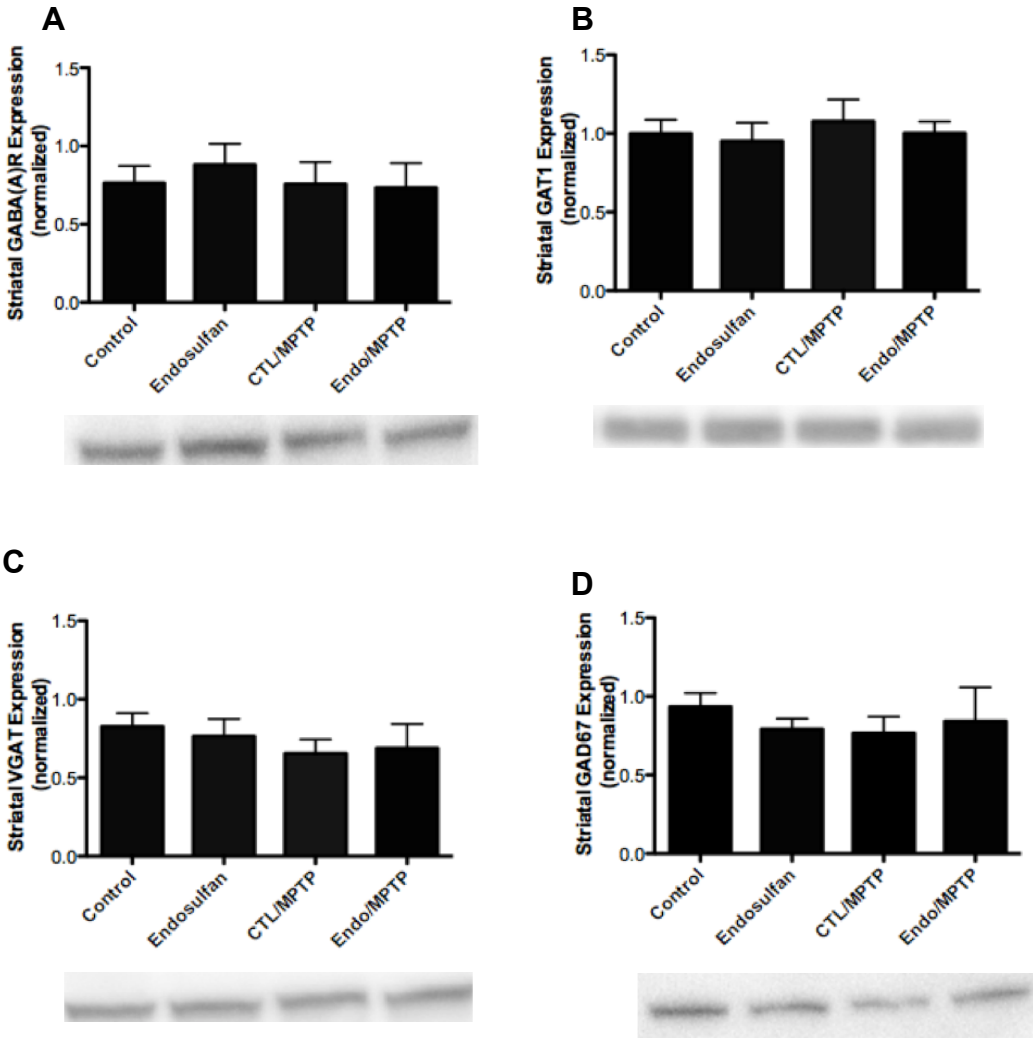

Figure 6. Western blot analysis of striatal GABAergic markers. Mice exposed to endosulfan I at concentrations of 0 or 1 mg/kg for 30 days and challenged with 2 doses of either saline or 10 mg/kg of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) show no effect on striatal GABAergic markers. One-way ANOVA and Newman-Keuls multiple comparison post-test was used for statistical analyses; n=5 per each treatment group. Data represent average $\pm$ SEM normalized to actin.

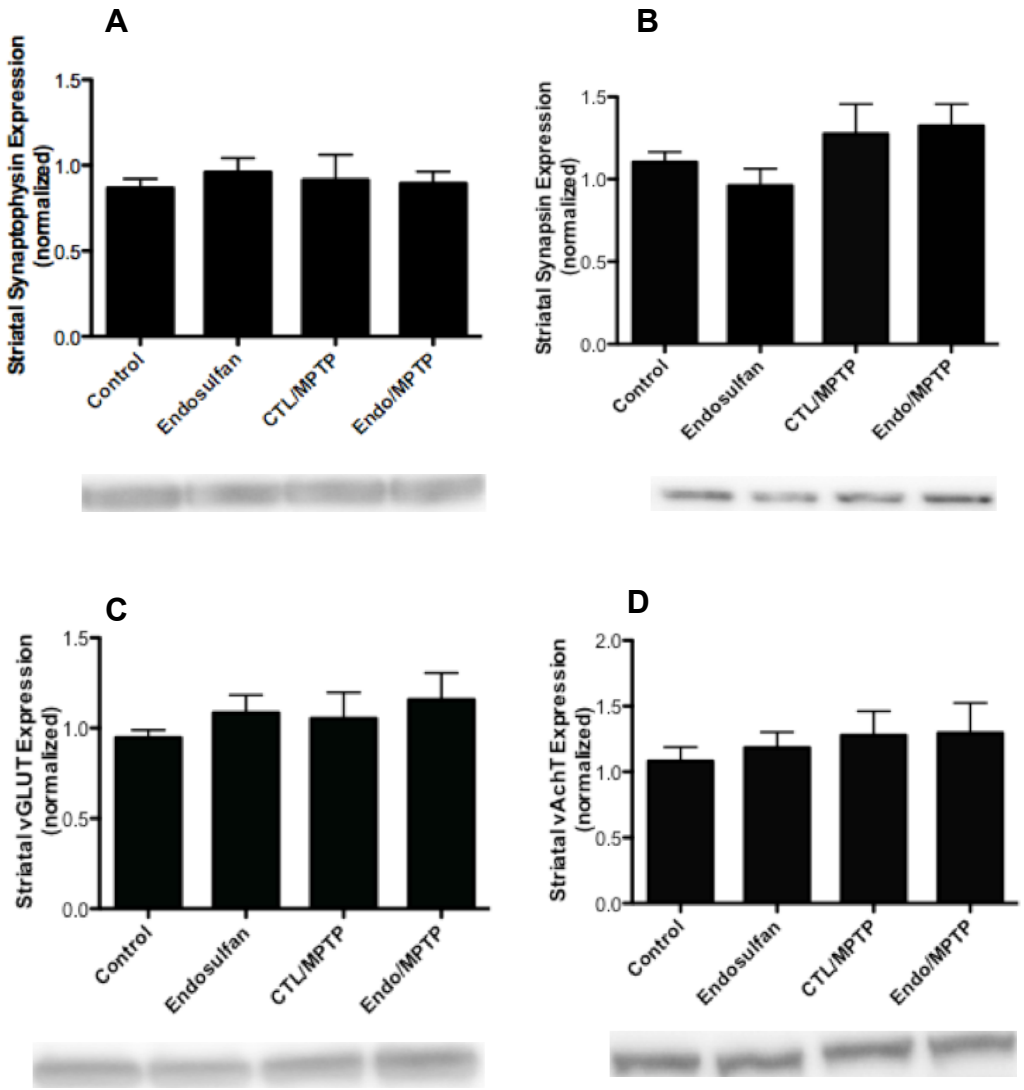

Figure 7. Western blot analysis of striatal synaptic markers and transporters. Mice exposed to endosulfan I at concentrations of 0 or 1 mg/kg for 30 days and challenged with 2 doses of either saline or 10 mg/kg of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) show no effect on striatal transporters or synaptic markers. One-way ANOVA and Newman-Keuls multiple comparison post-test was used for statistical analyses; n=5 per each treatment group. Data represent average $\pm$ SEM normalized to actin.

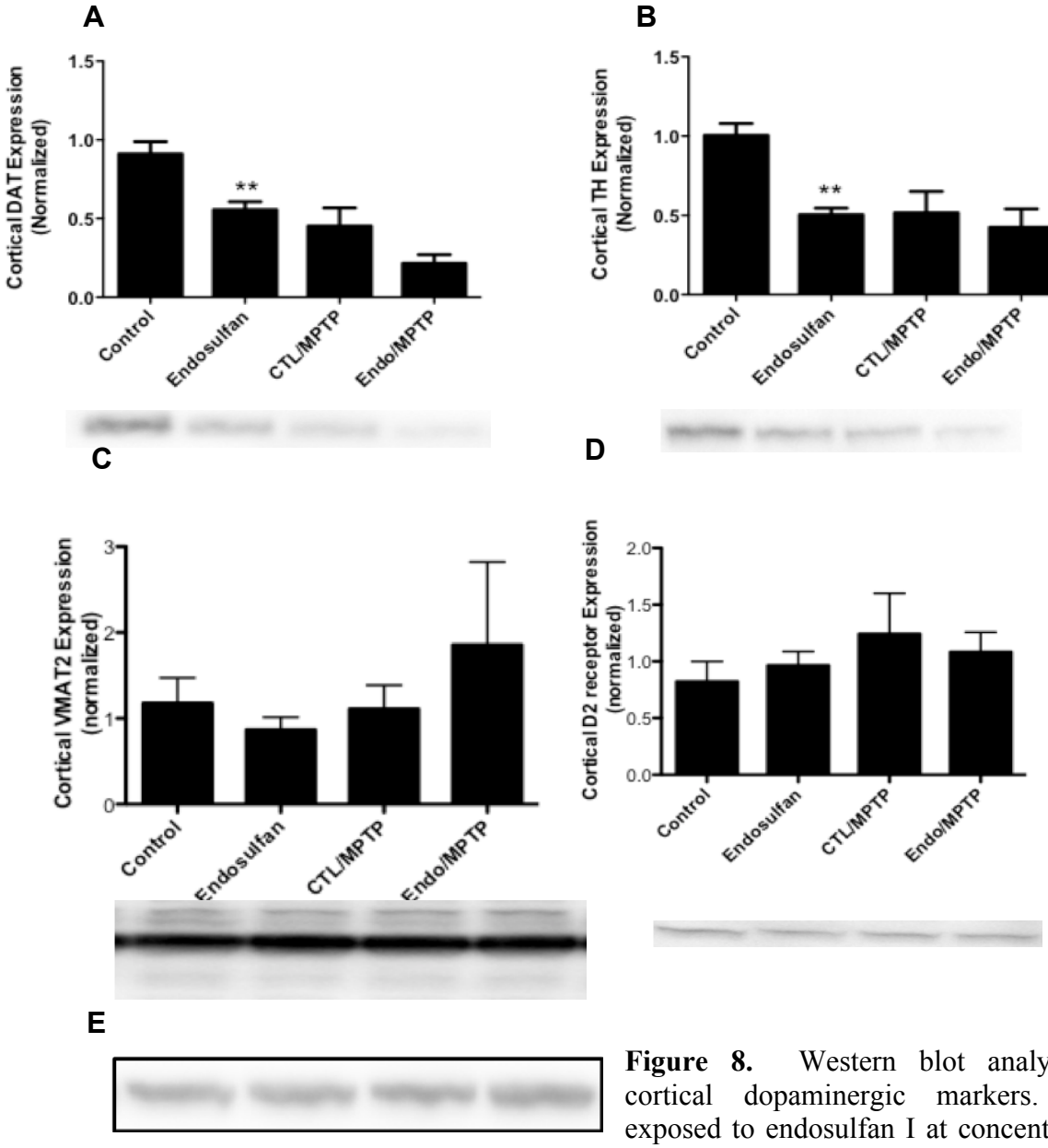

Figure 8. Western blot analysis of cortical dopaminergic markers. Mice exposed to endosulfan I at concentrations of 0 or 1 mg/kg for 30 days and challenged with 2 doses of either saline or 10 mg/kg of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) show a reduction in some cortical dopaminergic protein levels. One-way ANOVA and Newman-Keuls multiple comparison post-test was used for statistical analyses; n=5 per each treatment group. Data represent average $\pm$ SEM normalized to actin. A. Western blot analysis shows a significant reduction in cortical DAT levels in animals exposed to endosulfan as well as MPTP (** indicates $p < .01$). B. Western blot analysis shows a significant reduction in cortical TH levels in animals exposed to endosulfan as well as MPTP (** indicates $p < 01$). C. Western blot analysis shows no significant reduction in cortical VMAT2 expression by endosulfan or MPTP. D. Western blot analysis shows no significant reduction in cortical D2 receptor expression by endosulfan or MPTP. E. Representative Western blot of actin to ensure equal loading.

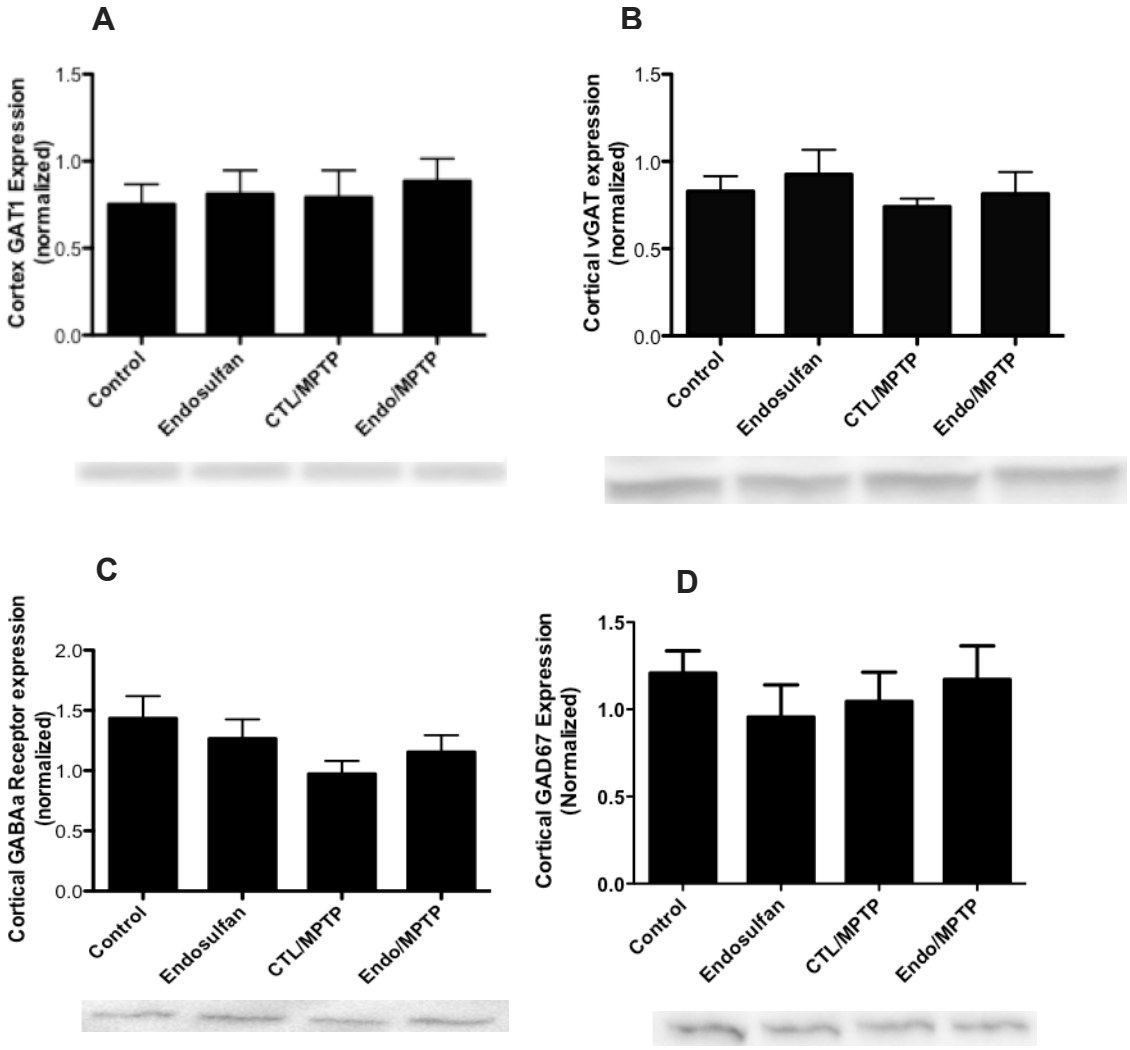

Figure 9. Western blot analysis of cortical GABAergic markers. Mice exposed to endosulfan I at concentrations of 0 or 1 mg/kg for 30 days and challenged with 2 doses of either saline or 10 mg/kg of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) show no effect on cortical GABAergic markers. One-way ANOVA and Newman-Keuls multiple comparison post-test was used for statistical analyses; n=5 per each treatment group. Data represent average $\pm$ SEM normalized to actin.

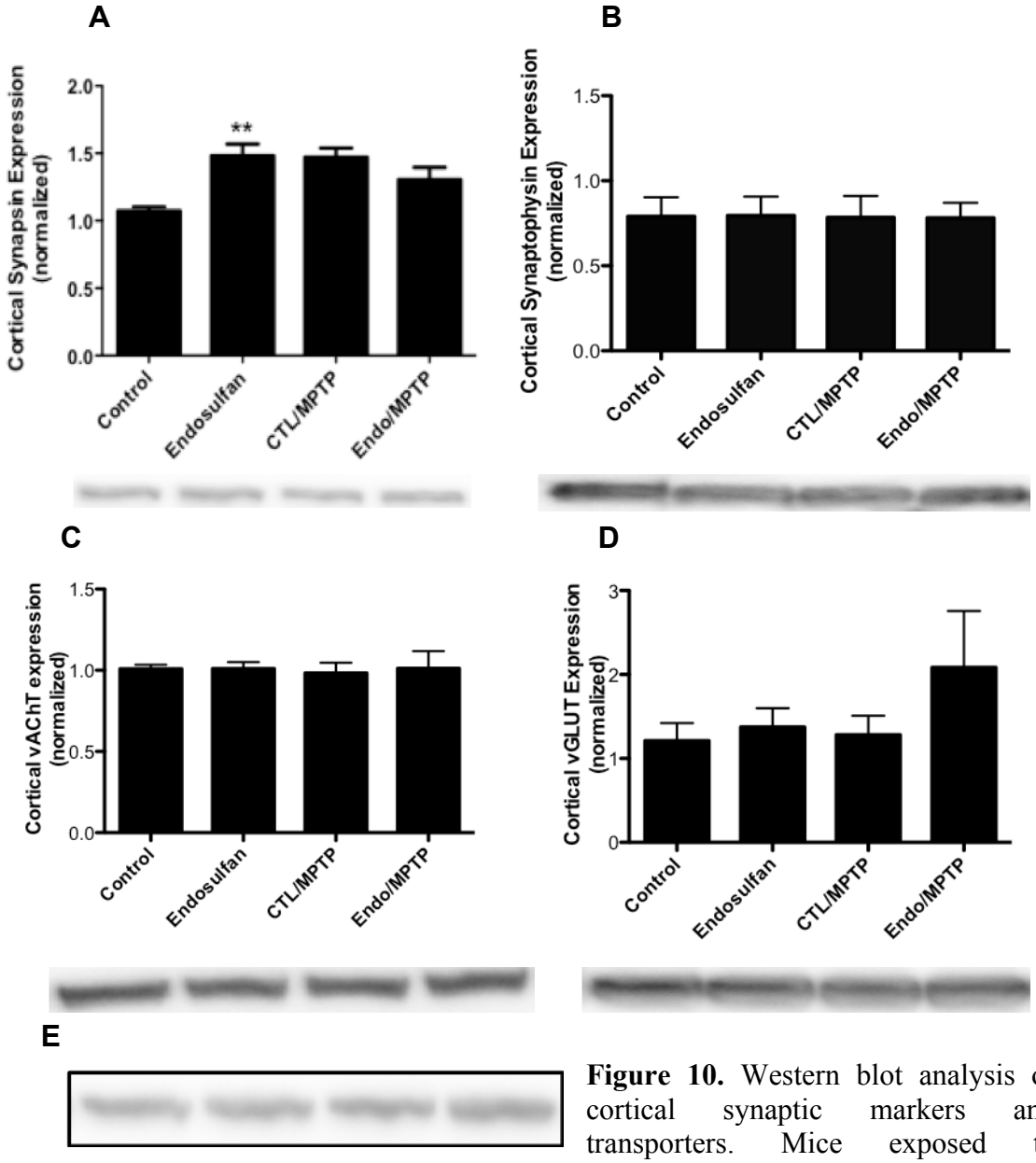

Figure 10. Western blot analysis of cortical synaptic markers and transporters. Mice exposed to endosulfan I at concentrations of 0 or 1 mg/kg for 30 days and challenged with 2 doses of either saline or 10 mg/kg of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) show no effect on cortical transporters by do impact a cortical synaptic marker. One-way ANOVA and Newman-Keuls multiple comparison post-test was used for statistical analyses; n=5 per each treatment group. Data represent average $\pm$ SEM normalized to actin. A. Western blot analysis shows a significant increase in cortical synapsin expression by endosulfan, although MPTP elicits no effect (** indicates $p<.01$). B. Western blot analysis shows no significant change in cortical synapsin expression by endosulfan or MPTP. C. Western blot analysis shows no significant change in cortical vAChT expression by endosulfan or MPTP. D. Western blot analysis shows no significant change in cortical vGLUT expression by endosulfan or MPTP. E. Representative Western blot of actin to ensure equal loading.

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